The *MYCN* Enigma: Significance of *MYCN* Expression in Neuroblastoma

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Abstract

***MYCN* amplification strongly predicts adverse outcome of neuroblastoma. However, the significance of *MYCN* expression in the clinical and biological behavior of neuroblastoma has been unclear. To address this question, we first examined the expression of *MYCN* in combination with *TrkA* (a favorable prognostic indicator of neuroblastoma) in 91 primary neuroblastoma by quantitative reverse transcription-PCR and investigated the relationship among patient survival, *MYCN*, and *TrkA* expressions. Three subsets of neuroblastoma were defined based on *MYCN* and *TrkA* expression. Neuroblastoma expressing the highest level of *MYCN* but little *TrkA* were *MYCN*-amplified cases, which had a 5-year survival of 9.3%. Interestingly, *MYCN* and *TrkA* expression showed a linear correlation ($r = 0.5664$, $P < 0.00005$) in neuroblastoma lacking *MYCN* amplification, and the 5-year survival of neuroblastoma patients with low *MYCN* and low *TrkA* expressions was 63.7%, whereas those with high expression of both had a 5-year survival of 88.1% ($P < 0.00005$). This nonlinear distribution of disease outcome relative to *MYCN* expression in neuroblastoma explains why *MYCN* expression is not predictive of neuroblastoma disease outcome by dichotomous division of the neuroblastoma cohort. However, high-level *MYCN* expression is associated with favorable outcome in neuroblastoma lacking *MYCN* amplification. Furthermore, forced expression of MYCN significantly suppresses growth of neuroblastoma cells lacking *MYCN* amplification by inducing apoptosis and enhancing favorable neuroblastoma gene expression. Collectively, these data suggest that high-level *MYCN* expression in neuroblastoma lacking *MYCN* amplification results in a benign phenotype. Thus, the high *MYCN* expression confers the opposite biological consequence in neuroblastoma, depending on whether or not *MYCN* is amplified.** (Cancer Res 2006; 66(5): 2826-33)

Introduction

Neuroblastoma is a common pediatric solid tumor of neural crest origin. The tumor occurs in infants and young children with primary sites in adrenal glands or the sympathetic chain. Neuroblastoma is unique because of its clinical bipolarity. It comes in two very different forms: favorable or unfavorable (1). Several prognostic markers have been described to predict the outcome of neuroblastoma, including the age of the patient at diagnosis, tumor stage, Shimada histology, DNA ploidy, serum ferritin or lactate dehydrogenase levels, and *MYCN* amplification (2–7). There are additional genetic and biological markers of neuroblastoma that are predictive of disease outcome. These include deletion or allelic loss of chromosome 1p or 11q (8–11), allelic gain of 17q (12), and the expression of transcripts encoding receptor tyrosine kinases (*TrkA* and *EPHB6*) and cell surface molecules (*CD44, EFNB2*, and *EFNB3*). These five genes in particular have been defined as favorable neuroblastoma genes, because high-level expression of these genes not only predicts favorable outcome of neuroblastoma clinically but also suppresses growth of unfavorable neuroblastoma cells *in vitro* and in mouse xenograft models (13–17). In addition, when one of the favorable neuroblastoma genes is expressed at high levels, patient disease outcome is favorable (15).

Among the prognostic indicators of neuroblastoma, *MYCN* amplification is strongly associated with advanced disease stages, rapid tumor progression, and the worst disease outcome (7). *MYCN* amplification occurs in about 20% to 25% of all neuroblastoma cases, and amplification of *MYCN* leads to its overexpression at both the mRNA and protein levels (18–22). It has been postulated that high-level expression of the MYCN protein in neuroblastoma results in activation of genes associated with aggressive tumor behavior (23). Nonetheless, the question as to whether *MYCN* expression is predictive of disease outcome of neuroblastoma remains controversial (13, 19–21, 24–29); thus, the significance of *MYCN* expression in neuroblastoma has been unclear.

In this study, we have attempted to approach this controversy by analyzing *MYCN* expression in relation to *TrkA* expression (a well-established favorable marker of neuroblastoma) in a cohort of 91 neuroblastoma tumor specimens. This approach enabled us to identify a nonlinear relationship between disease outcome of neuroblastoma and *MYCN* expression, which in turn explains why *MYCN* expression cannot predict neuroblastoma disease outcome by dichotomous division of the overall study cohort. Interestingly, our clinical observations also suggest that high-level *MYCN* expression is a favorable feature of neuroblastoma lacking *MYCN* amplification, including those of advanced stages. In addition, we showed experimentally that forced expression of MYCN resulted in the reduction in the viability of neuroblastoma cells lacking *MYCN* amplification. Moreover, this growth suppressive effect of MYCN was in part due to apoptosis and the enhancement of favorable neuroblastoma gene expression. Together, these observations suggest that high-level expression of *MYCN* in neuroblastoma

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-05-0854

lacking *MYCN* amplification results in a benign phenotype. Thus, the high *MYCN* expression confers the opposite biological consequence in neuroblastoma, depending on whether or not *MYCN* is amplified.

Materials and Methods

Primary neuroblastoma tumor samples. The 91 neuroblastoma tumor specimens included those obtained from the Tumor Bank of The Children's Hospital of Philadelphia, The Tumor Bank of The Pediatric Oncology Group, The Tumor Bank of The Children's Cancer Group, and Memorial Sloan-Kettering Cancer Center. These included 16 stage I tumors, 16 of stage II, 9 of stage IVS, 19 of stage III, and 31 stage IV tumors. Among these tumors, 2 stage III tumors and 13 stage IV tumors had *MYCN* amplification. In this cohort, 77% of tumor specimens were from the former Children's Cancer Group institutions, whereas 23% of them were from the former Pediatric Oncology Group institutions. The median follow-up of this neuroblastoma cohort was 5.0 years, and the overall survival was 69.7%. Four established prognostic factors, including age at diagnosis, stage, *MYCN* amplification, and *TrkA* expression, were used to verify the neuroblastoma cohort by single variable Cox regression analysis. The use of human tumor samples for the study was reviewed and approved by the institutional review board.

RNA extraction and quantitative reverse transcription-PCR. Experimental procedures for RNA preparation, reverse transcription, and quantitative reverse transcription-PCR (RT-PCR) have been previously described elsewhere (15, 30, 31). Results of this quantitative RT-PCR were shown to be consistent with those obtained by Northern blot analysis (31, 32).

Statistical analysis. Differential expression of variables in given subgroups of neuroblastoma was compared by *t* tests. Survival probabilities in various subgroups were estimated according to the methods of Kaplan and Meier (33). Survival distributions were compared using log-rank tests (34). Cox regression analysis (35) was also used to assess the prognostic significance of variables. $P < 0.05$ was considered statistically significant.

***In vitro* experiments.** Two human neuroblastoma cell lines (SY5Y and SK-N-AS) were used in this study because they lack *MYCN* amplification. Transient transfection was carried out by electroporation (120 V, 25 m second square wave) using 0.2-cm cuvettes and a Bio-Rad Xcell electroporator. Five-microgram DNA was used to transfect 10^7 cells. SK-N-AS cells were also transfected with pBABE (vector control) or pBABE carrying a *MYCN-ER* fusion construct (gifts from Drs. Antonio Iavarone and Anna Lasorella, Columbia University, New York, NY) by retrovirus transduction. The *ER* portion of the construct contains a point mutation, which makes the ER only responsive to 4-hydroxyl tamoxifen (4-OHT; ref. 36). Stable SK-N-AS transfectants were obtained after the selection with 1 μg/mL puromycin. The *MYCN-ER* transfectants were further subjected to limiting dilution, and clones with high MYCN-ER expression were identified by Western blot analysis. MYCN protein was detected with the mouse monoclonal antibody, NCM II 100 (37), whereas pro and active forms of caspase-3 were detected by rabbit polyclonal antibodies (AAP113; Stressgen, San Diego, CA). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt assay (Promega, Madison, WI) was carried out according to the manufacturer's instructions.

Results

Pattern of *MYCN* expression in neuroblastoma with respect to tumor stage and age at diagnosis. To gain insight into the prognostic significance of *MYCN* expression in neuroblastoma, we first examined whether *MYCN* expression was associated with age or stage in the overall neuroblastoma ($n = 91$). *MYCN* expression was associated with tumor stage ($P = 0.0402$) but not with age at diagnosis ($P = 0.5469$), although 14 of the 15 *MYCN*-amplified cases in the group were >1 year of age. In contrast to *MYCN*, high *TrkA* expression was significantly associated with both low-stage tumors (stages I, II, and IVS; $P = 0.0006$) and younger age (<1 year; $P < 0.0001$; data not shown). When *MYCN*-nonamplified neuroblastoma were analyzed separately ($n = 76$), higher *MYCN* and *TrkA* expressions were both associated with the younger age ($P = 0.0048$ and $P = 0.0006$, respectively), whereas neither *MYCN* nor *TrkA* expression was associated with stage ($P = 0.2505$ and $P = 0.0592$, respectively; see Supplementary Data).

Whether *MYCN* expression predicts outcome of neuroblastoma depends on how the cohort is dichotomized. Previous studies on the prognostic significance of *MYCN* expression in neuroblastoma have been controversial. We therefore reexamined this issue by performing Kaplan-Meier analysis using different cutoff values. As shown in Fig. 1, whether *MYCN* expression predicts outcome of neuroblastoma depended on how the cohort was dichotomized. *MYCN* expression was prognostic when the lowest value among the *MYCN*-amplified cases was used as the cutoff value ($P = 0.0001$; Fig. 1*A*). However, when the median value

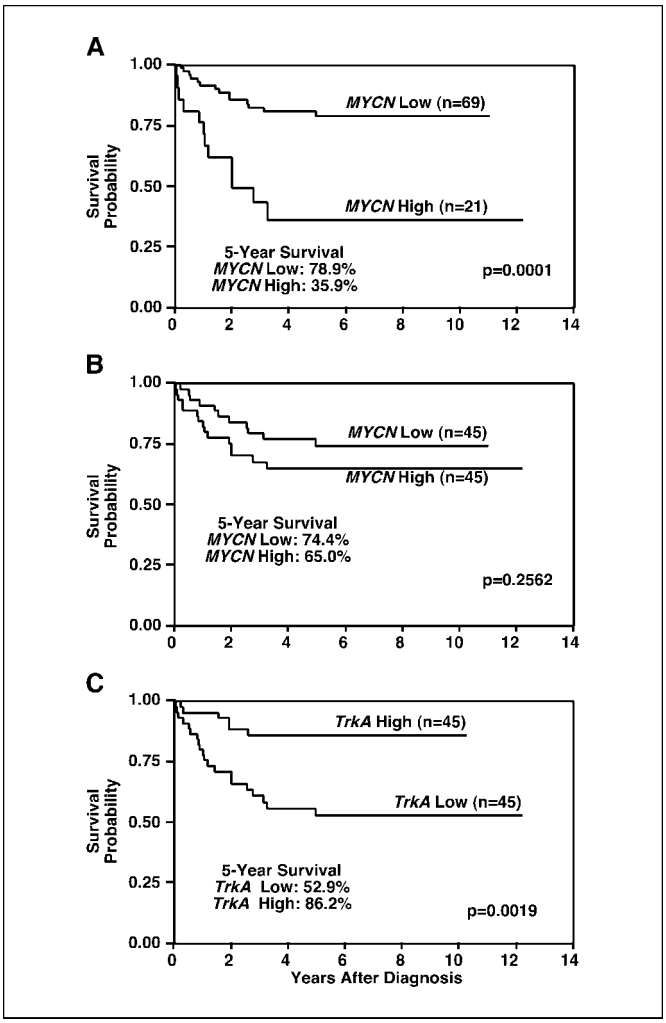

Figure 1. *MYCN* and *TrkA* expression and survival of neuroblastoma patients. Kaplan-Meier survival analysis was used to assess the survival probabilities of neuroblastoma subsets defined by levels of *MYCN* or *TrkA* expression. The log-rank test was used to assess difference in survival of the neuroblastoma subsets indicated. *MYCN* expression levels ranged from 0.03 to 4.41 in the study cohort, whereas *TrkA* expression levels ranged from 0.007 to 3.21. *A,* the entire cohort of neuroblastoma was dichotomized using the lowest value of *MYCN* expression in the *MYCN*-amplified neuroblastoma (2.0). *B,* the cohort of neuroblastoma was dichotomized using the median value of *MYCN* expression (1.529). *C,* the cohort of neuroblastoma was dichotomized using the median value of *TrkA* expression (1.628). Survival data was available for 90 of the 91 neuroblastoma cases examined.

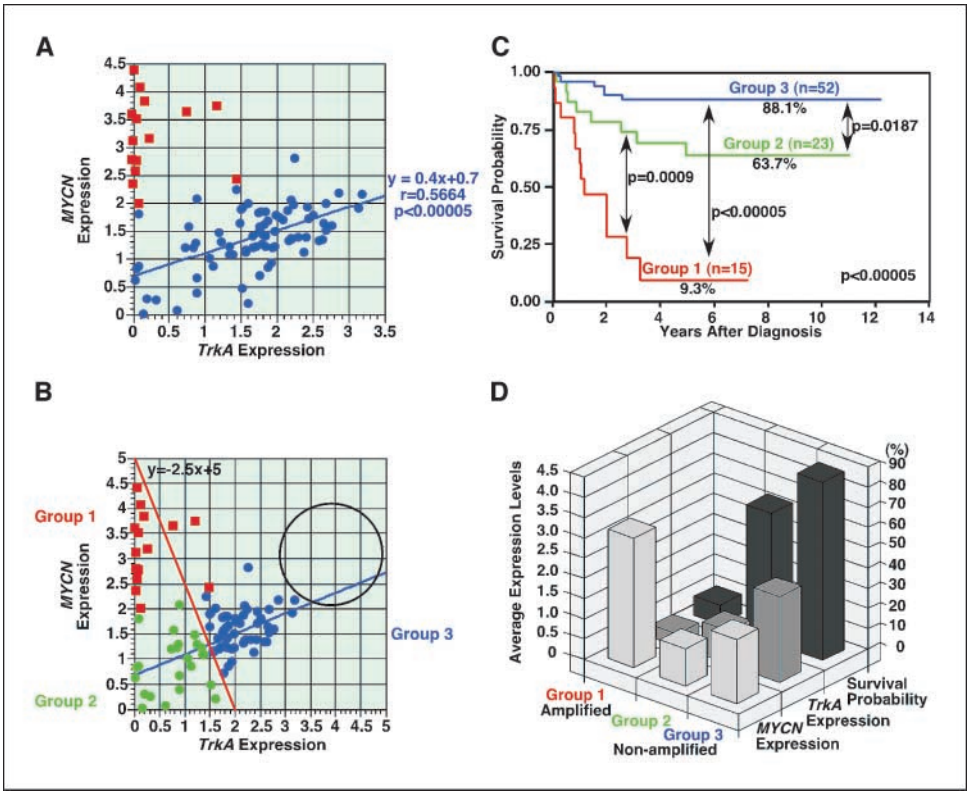

Figure 2. Relationship between *MYCN* and *TrkA* expression in neuroblastomas. *A,* relationship between *MYCN* and *TrkA* expression in the overall neuroblastoma population ($n = 91$). Expression levels of *MYCN* were plotted against those of *TrkA* in the corresponding tumors. *Red squares,* neuroblastoma with *MYCN* amplification ($n = 15$). *Blue circles,* neuroblastoma without *MYCN* amplification ($n = 76$). *MYCN* expression positively correlates with *TrkA* expression in neuroblastoma lacking *MYCN* amplification, and a linear regression analysis yielded the linear regression formula of $y = 0.4x + 0.7$. Pearson correlation coefficient and *P* were then calculated. *B,* to determine the cutoff line (*red*) that dichotomizes the neuroblastoma lacking *MYCN* amplification, the half value of the highest *TrkA* expression was first calculated, which was 1.6. The perpendicularly intersecting line against the linear regression line at this point was drawn and was adjusted to identify the cutoff point (1.5) that clearly dichotomizes this neuroblastoma population. *Left of intersecting line, green circles,* low *TrkA* and low *MYCN* group ($n = 24$; group 2). *Right of intersecting line, blue circles,* high *TrkA* and high *MYCN* group ($n = 52$; group 3). Neuroblastoma with *MYCN* amplification correspond to group 1 (*red squares*), which express highest levels of *MYCN* and little *TrkA* ($n = 15$). *Black circle,* putative neuroblastoma that were not detected clinically. *C,* Kaplan-Meier survival analysis was used to assess the survival probabilities of three neuroblastoma subsets defined by the patterns of *MYCN* and *TrkA* expression indicated in (*B*). Ninety of the 91 neuroblastoma cases in the cohort had survival data. The log-rank test was used to assess difference in survival of the neuroblastoma subsets. *D,* three-dimensional graphic representation of the relationship among *MYCN* expression (*light gray*), *TrkA* expression (*medium gray*), and patient survival (*dark gray*). *Columns,* mean values of *MYCN* and *TrkA* expression and the survival rates of three groups.

was used as the cutoff, *MYCN* expression was no longer prognostic ($P = 0.2562$; Fig. 1*B*). In contrast, neuroblastoma with high *TrkA* expression showed a significant survival advantage over those with low *TrkA* expression when the median value was used as the cutoff value ($P = 0.0019$; Fig. 1*C*). Moreover, *TrkA* expression yielded the same result regardless of the cutoff value that was used to dichotomize the study cohort (data not shown).

Combination of *MYCN* and *TrkA* expression defines three neuroblastoma subsets with distinct survival probabilities. We previously found that *MYCN* and *TrkA* expression (a favorable prognostic indicator of neuroblastoma) correlated with each other in a cohort of low-stage neuroblastoma (38). This observation suggests that by investigating the expression of *MYCN* in relation to *TrkA* expression, one may gain a better understanding of the prognostic significance of *MYCN* expression in neuroblastoma. As shown in Fig. 2*A*, neuroblastoma expressing very high levels of *MYCN* but little *TrkA* were *MYCN*-amplified cases (shown in *red squares*). On the other hand, there was a positive linear correlation between *MYCN* expression and *TrkA* expression in neuroblastoma lacking *MYCN* amplification ($r = 0.5664$, $P < 0.00005$; Fig. 2*A, blue circles*). Based on *MYCN-TrkA* expressions, we further divided neuroblastoma lacking *MYCN* amplification into two subsets, shown in *green* (low *MYCN* and low *TrkA*) and *blue* (high *MYCN* and high *TrkA*; Fig. 2*B*). Thus, this yielded three subsets of neuroblastoma with distinct levels of *MYCN* and *TrkA* expression: group 1, group 2, and group 3 (Fig. 2*B*). It should be noted that no neuroblastoma was found in the area (indicated by the *black circle*) that represents *MYCN* nonamplified neuroblastoma expressing high *MYCN* levels compatible to those in *MYCN*-amplified cases.

As shown in Fig. 2*C*, the Kaplan-Meier analysis showed that the survival of group 1 was the worst (5-year survival of 9.3%), whereas group 2 showed a 5-year survival rate of 63.7%. In contrast, group 3 had a 5-year survival rate of 88.1%. Moreover, the differences in a 5-year survival among the three neuroblastoma subsets were statistically significant (Fig. 2*C*). When the 5-year survival rates of the three neuroblastoma groups were plotted against the mean value of *MYCN* or *TrkA* expression of each group (Fig. 2*D*), it was evident that the level of *MYCN* expression did not correlate with survival rates of the neuroblastoma subsets, whereas *TrkA* expression did. In neuroblastoma lacking *MYCN* amplification, however, neuroblastoma with low *MYCN* expression (group 2; Fig. 2*C* and *D*) was associated with a lower survival rate than that of the *MYCN* high subset (group 3; Fig. 2*C* and *D*), and such a difference in survival rate was statistically significant ($P = 0.0187$; Fig. 2*C*).

***MYCN* expression alone can predict disease outcome of neuroblastoma if the cohort is properly stratified.** The results shown in Fig. 2*C* and *D* suggest that *MYCN* expression alone would predict neuroblastoma disease outcome if the cohort is properly stratified. In fact, our data revealed that *MYCN* expression itself was significantly predictive of disease outcome of the entire neuroblastoma cohort by trichotomization (Fig. 3*A*). Moreover, *MYCN* expression predicted disease outcome of neuroblastoma lacking *MYCN* amplification by dichotomization (Fig. 3*B*). In addition, as shown in Fig. 4, Kaplan-Meier analyses identified trends that low *MYCN* expression was associated with poor disease outcome of *MYCN*-nonamplified neuroblastoma of advanced stage (n = 36, P = 0.0060), of age over 1 year (n = 45, P = 0.0673), and of advanced stage and age over 1 year (n = 25, P = 0.0801). These results suggest that high-level *MYCN* expression is a favorable but not an unfavorable feature of high-risk neuroblastoma lacking *MYCN* amplification.

Forced expression of MYCN reduced the viability of neuroblastoma cells lacking *MYCN* amplification by inducing apoptosis and enhancing favorable neuroblastoma gene expression. The above observations collectively suggest that high-level *MYCN* expression in neuroblastoma lacking *MYCN* amplification results in a benign phenotype. To test this idea, we transfected two neuroblastoma cell lines lacking *MYCN* amplification (SY5Y and SK-N-AS expressing little endogenous MYCN) with a *MYCN* expression construct or a vector control and examined the effect of MYCN on cell growth. As shown in Fig. 5*A*, an increase in MYCN expression caused a significant reduction in the viability of these neuroblastoma cells, which was accompanied by an increase in apoptosis assessed by caspase-3 activation (Fig. 5*B*). Moreover, forced expression of MYCN in SY5Y and SK-N-AS resulted in an enhanced expression of favorable neuroblastoma genes (*EFNB3* in SY5Y and *CD44* in both cell lines; Fig. 5*C*). Nonetheless, *TrkA* expression was not increased in these *MYCN* transfectants (data not shown; see Discussion). It should be mentioned that all the *MYCN*-transfected cells eventually died off under the selection condition to maintain high MYCN expression levels, and no stable *MYCN* transfectant was established.

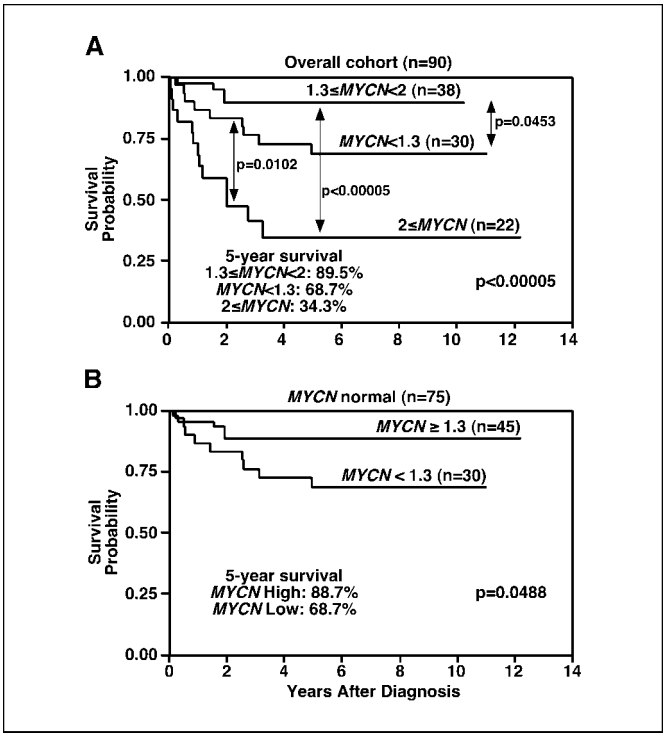

Figure 3. *MYCN* expression is predictive of the overall neuroblastoma cohort by trichotomization and subsets of neuroblastoma lacking *MYCN* amplification by dichotomization. *A,* Kaplan-Meier survival analysis was used to assess the survival probabilities of three neuroblastoma subsets with different *MYCN* expression levels. The cutoff values of 2 and 1.3 were used to trichotomize the study cohort as indicated (n = 90). The value 2 corresponds to the lowest value among the *MYCN*-amplified cases, whereas 1.3 corresponds to the *MYCN* expression value at the cross-section of the two intersecting lines in Fig. 2*B*. *B,* neuroblastoma cases lacking *MYCN* amplification (n = 75) was dichotomized based on the *MYCN* expression value of 1.3. Survival of each group was assessed by Kaplan-Meier analysis. The log-rank test was used to assess difference in survival of the neuroblastoma subsets.

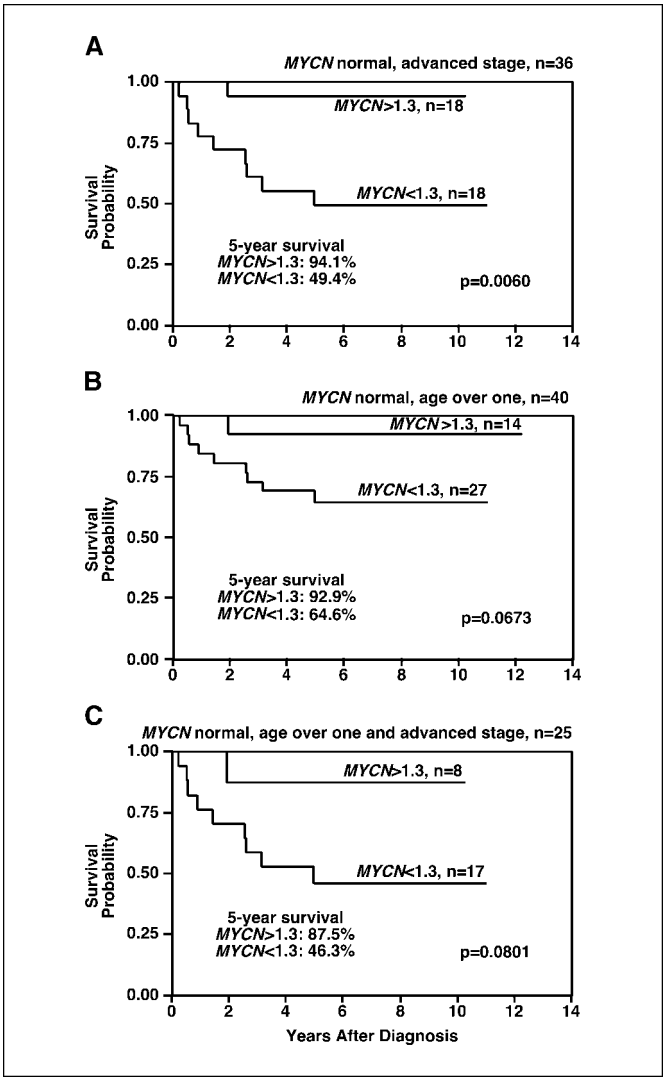

Figure 4. Low *MYCN* expression tends to associate with a poor disease outcome of unfavorable neuroblastoma without *MYCN* amplification. Kaplan-Meier survival analysis was employed to assess survival probabilities of *MYCN*-nonamplified neuroblastoma with unfavorable features (advanced stage and/or age over 1 year) by *MYCN* expression. The cutoff value 1.3 was used to dichotomize the study cohort as indicated. *A,* low *MYCN* expression was significantly associated with a poor disease outcome of advanced-stage neuroblastoma without *MYCN* amplification (n = 36). A similar trend was also observed for *MYCN*-nonamplified neuroblastoma with age over 1 year (n = 40; *B*) and with age over 1 year and advanced-disease stage (n = 25; *C*).

To address the effect of sustained high-level MYCN expression in neuroblastoma cells lacking *MYCN* amplification, we transfected several neuroblastoma cell lines lacking *MYCN* amplification with pBABE vector or pBABE carrying a *MYCN-ER* construct. The MYCN-ER is only conditionally active in its transactivation activity (36) when the ER domain is bound by its ligands. Among the cell lines tested, only SK-N-AS gave rise to clones with high MYCN-ER expression after the drug selection and limiting dilution. These results suggest that even the closed conformation of MYCN-ER (with no ligand present) has a marked effect on the viability of the neuroblastoma cells (see below).

As shown in Fig. 6*A*, the introduction of MYCN-ER caused a significant growth suppression of SK-N-AS cells, and 4-OHT further augmented the growth suppressive activity of MYCN-ER, whereas growth of SK-N-AS cells transfected with pBABE was unaffected by the addition of 4-OHT. These results suggest that the transactivation activity is not absolutely required for the growth suppressive effect of MYCN-ER on *MYCN*-nonamplified neuroblastoma cells, or alternatively, the MYCN-ER produced in SK-N-AS cells is partially active without ligand binding. Interestingly, there was a significant enhancement of *EPHB6* and *CD44* expression in SK-N-AS transfected with *MYCN-ER* (Fig. 6*B*). In the presence of 4-OHT, the enhancement of *EPHB6* expression was slightly reduced, but the expression of *CD44* was further enhanced (Fig. 6*B*). These results suggest that favorable neuroblastoma genes play an important role in growth suppressive effect of MYCN on neuroblastoma cells lacking *MYCN* amplification.

Discussion

MYCN amplification was identified in neuroblastoma by Schwab et al. over 20 years ago (39). Soon after this discovery, *MYCN* amplification was confirmed as the most significant prognostic indicator of adverse disease outcome in neuroblastoma (7, 40). Subsequently, a series of studies have been conducted to address the prognostic significance of *MYCN* expression in neuroblastoma (13, 19–21, 24–29). Conclusions from these studies, however, have been inconsistent to date. This study was undertaken to address this controversy and to gain insight into biological functions of *MYCN* in neuroblastoma.

As mentioned, the results of Kaplan-Meier analyses indicate that whether *MYCN* expression predicts neuroblastoma disease outcome depending on which cutoff values are used to dichotomize the neuroblastoma study cohort (Fig. 1*A* and *B*). These observations suggest that survival of neuroblastoma patients does not simply correlate with levels of *MYCN* expression in the overall neuroblastoma population and cast doubt on the previous assumption that *MYCN* expression correlates with disease outcome of neuroblastoma. Our study provides evidence that this assumption is in fact incorrect (Fig. 2*D*). The relationship between *MYCN* expression and disease outcome in the overall neuroblastoma population is nonlinear, and this explains why standard survival analyses, two-arm Kaplan-Meier or Cox regression analysis, cannot yield the correct conclusion as to the prognostic significance of *MYCN* expression in neuroblastoma. This fact has not been discussed in the

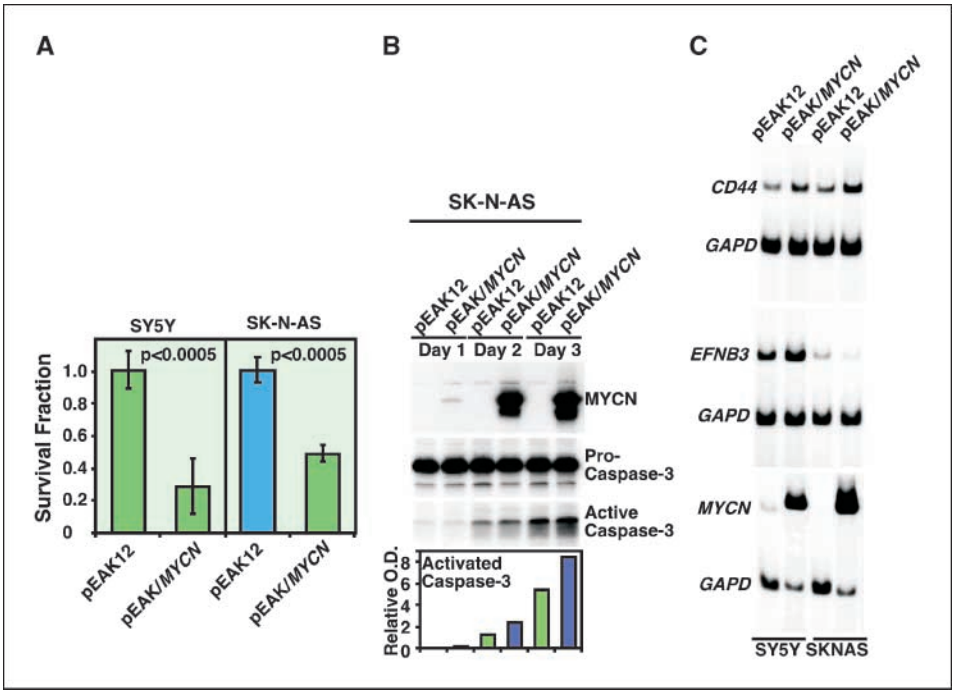

Figure 5. High-level expression of MYCN in neuroblastoma cell lines lacking *MYCN* amplification results in growth suppression, an increase in apoptosis, and enhanced favorable neuroblastoma gene expression (*EFNB3* and *CD44*). *A,* SY5Y and SK-N-AS cell lines were chosen for this analysis because they lack *MYCN* amplification. SY5Y and SK-N-AS cells were transfected with either pEAK12 vector or the vector containing a *MYCN* cDNA by electroporation. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt assay was done to determine the effect of MYCN overexpression on the viability of SY5Y and SK-N-AS cells. After transfection, the neuroblastoma cells were cultured in 24-well plates. Puromycin (1 μg/mL) was added as the selection drug 24 hours after the transfection. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt assay was done on day 6. *B,* caspase activation was investigated in *MYCN*-transfected SK-N-AS cells by Western blot analysis using antibodies that could detect both pro and active forms of caspase-3. The drug selection (puromycin at 1 μg/mL) was started at 24 hours after of transfection, which killed nontransfected cells. The baseline increase in caspase-3 activity was due to the drug selection process. *C,* SY5Y and SK-N-AS cells were transiently transfected with pEAK12 vector or the vector carrying a *MYCN* cDNA. Forty-eight hours after the transfection, puromycin at 0.5 μg/mL was added to the culture to select the transfectants. The cells were harvested 48 hours after the drug selection. Gene expression studies were done as described in Materials and Methods.

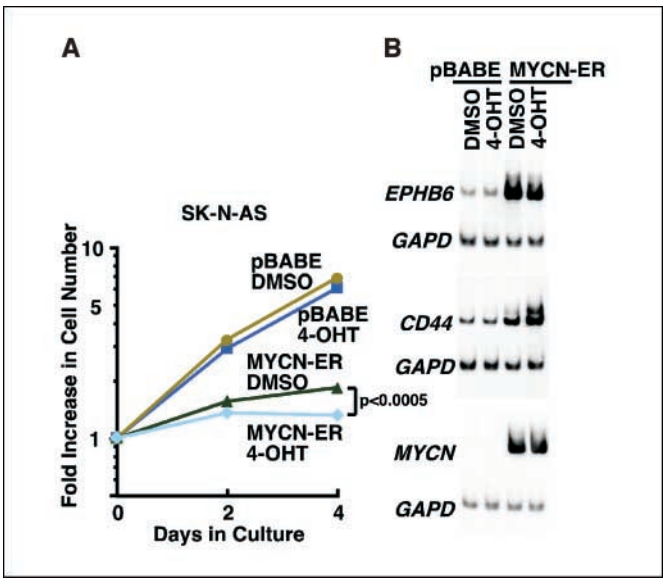

Figure 6. Sustained high-level MYCN expression in neuroblastoma cells without *MYCN* amplification causes growth suppression and an increase in favorable neuroblastoma gene expression (*EPHB6* and *CD44*). SK-N-AS cells were transfected with pBABE (vector control) or pBABE carrying a *MYCN-ER* fusion construct by retrovirus transduction. Stable transfectants were obtained after the selection with 1 μg/mL puromycin. The *MYCN-ER* transfectants were further subjected to limiting dilution, and clones with high MYCN-ER expression were identified by Western blot analysis using a MYCN-specific monoclonal antibody, NCM II 100. *A,* SK-N-AS/pBABE and SK-N-AS/*MYCN-ER* cells were grown for up to 4 days in the presence of solvent control (DMSO) or 4-OHT (500 nmol/L), which induced an open (active) conformation of the MYCN-ER fusion protein. Cell growth was monitored by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt assay. The introduction of MYCN-ER itself caused a significant growth suppression of SK-N-AS cells, and 4-OHT further enhanced growth suppression of SK-N-AS/MYCN-ER cells. *B,* the expression of favorable neuroblastoma genes was examined in these transfectants with or without the 4-OHT treatments for 6 days.

previous studies (13, 19–21, 24–29), and it explains why these studies reached inconsistent conclusions.

The difference in the methodologies used may also have contributed to the discrepancy in the results of others and our group. β_2-Microglobulin transcripts were often used as an internal reference to assess *MYCN* transcript levels by quantitative RT-PCR in neuroblastoma (26, 28, 29). However, it is well recognized that most neuroblastoma express little or no β_2-microglobulin (41), except stage IVS neuroblastoma (42), and that β_2-microglobulin positive cells in the specimens of stage I to IV tumors are likely of nontumor origin, such as blood vessels and stromal tissues (43).[6] The quantity of these nontumor components in neuroblastoma specimens differs considerably from one case to another; therefore, the use of β_2-microglobulin transcripts as an internal reference in quantitative RT-PCR assay for neuroblastoma could lead to inaccurate results. In contrast, *GAPD* expression, which was used in this study, is relatively constant among low-stage and advanced-stage neuroblastoma (15, 38) and thus is more suitable for an internal reference in quantitative RT-PCR in gene expression studies of neuroblastoma.

Importantly, our study revealed that in neuroblastoma lacking *MYCN* amplification, neuroblastoma with low *MYCN* expression had a significantly lower survival rate than those with higher *MYCN* expression. This observation suggests that elevated *MYCN* expression provides neuroblastoma lacking *MYCN* amplification with a benign phenotype and may even lead to tumor regression. This may explain why we do not observe cases of neuroblastoma without *MYCN* amplification expressing very high levels of *MYCN* (Fig. 2*B*). In fact, our *in vitro* studies confirm that elevated expression of MYCN significantly reduced the viability of neuroblastoma cells lacking *MYCN* amplification through the induction of apoptosis and the enhancement of favorable neuroblastoma gene expression (Figs. 5 and 6). Moreover, pharmacologic augmentation of MYCN in SY5Y cells by proteasome inhibitors was associated not only with growth suppression[7] but also with enhanced expression of favorable neuroblastoma genes *EPHB6* and *CD44* (44). As previously defined, favorable neuroblastoma genes are genes whose high-level expression predicts favorable neuroblastoma disease outcome and suppresses growth of unfavorable neuroblastoma cells (15, 44). In this regard, it is of interest to note that *MYCN* can be considered a conditional favorable neuroblastoma gene in neuroblastoma lacking *MYCN* amplification. In addition, neuroblastoma cell lines without *MYCN* amplification, which express little or no MYCN, have often been used to assess the biological effect of high-level MYCN expression in neuroblastoma by its ectopic expression via transfection. This operation sensitizes these neuroblastoma cells to apoptosis with or without additional stimuli (45–48), and these results are even thought to be paradoxical (48). However, results of this study are in fact consistent with these previous experimental data.

Based on the positive correlation between *MYCN* and *TrkA* expression in neuroblastoma lacking *MYCN* amplification, one might ask whether forced expression of MYCN could result in an enhancement of *TrkA* expression in *MYCN*-nonamplified neuroblastoma cell lines. However, we have not been able to recapitulate this clinical observation *in vitro*. One possible explanation is that the TrkA promoter in these neuroblastoma cell lines may not be accessible for MYCN-mediated transactivation, because all E-boxes in the TrkA promoter region contain CpG in their cores and thus can be subjected to DNA methylation, which in turn makes them inaccessible for MYCN binding (49). To fully restore the expression of TrkA in these neuroblastoma cells, high-level MYCN as well as demethylation of the TrkA promoter may be required. DNA methylation of E-boxes in the TrkA promoter region also may be a general feature of unfavorable neuroblastoma, from which all neuroblastoma cell lines were derived. On the other hand, the corresponding E-boxes of the group 3 neuroblastoma (Fig. 2) may not be subjected to hypermethylation and therefore be accessible for MYCN-mediated transactivation. Because the group 3 neuroblastoma includes a significantly higher number of younger patients (59.6%) than the group 2 neuroblastoma (16.7%, $P < 0.001$), DNA methylation of E-boxes may be an age-dependent phenomenon. This explanation is in fact consistent with our observation that in *MYCN*-nonamplified neuroblastoma, higher *MYCN* and *TrkA* expression is associated with younger age of the patients (see Results and Supplementary Data).

The *MYCN*-transgenic mouse model has been suggested to represent human neuroblastoma, and this model has been employed to explain the effect of an elevated *MYCN* expression

[6] N. Ikegaki, unpublished observation.

[7] X. Tang and N. Ikegaki, unpublished observation.

on the clinical behavior of human neuroblastoma (50, 51). However, this model only resembles a small fraction of human neuroblastoma cases (i.e., paraspinal stage III neuroblastoma); therefore, one cannot generalize the effect of *MYCN* expression on human neuroblastoma based solely on this model. In addition, it is likely that *MYCN* is merely substituting for *MYC* in the *MYCN* transgenic mouse model, because human neuroblastoma cases lacking *MYCN* amplification generally express high levels of *MYC* but not *MYCN*. In fact, neuroblastoma cell lines lacking *MYCN* amplification express high levels of MYC but little or no MYCN with the exception of NBL-S, which expresses relatively high levels of MYCN but not MYC (52).

It is not clear how high MYCN expression exerts such differential effects on *MYCN*-amplified and *MYCN*-nonamplified neuroblastoma. However, enhanced expression of favorable neuroblastoma genes by MYCN in *MYCN*-nonamplified tumors is one possible mechanism. In addition, MYC family proteins are known to promote both cell proliferation and apoptosis depending on the cellular context (53). Perhaps, in neuroblastoma lacking *MYCN* amplification, the apoptosis-inducing function of MYCN is dominant. In contrast, in *MYCN*-amplified tumors, the balance between the two opposite effects may be shifted towards proliferation through mechanisms that protect these cells from MYCN-induced apoptosis. Taken together, our study suggests that an elevated *MYCN* expression results in two opposite biological consequences, depending on whether a neuroblastoma has *MYCN* amplification or not. High *MYCN* expression in *MYCN*-amplified neuroblastoma may confer a clinically aggressive phenotype, whereas high *MYCN* expression in *MYCN*-nonamplified neuroblastoma gives rise to a benign phenotype, including spontaneous tumor regression, one of the most intriguing characteristics of neuroblastoma.

Acknowledgments

Received 3/14/2005; revised 12/13/2005; accepted 12/22/2005.

Grant support: NIH grants CA97255 (X.X. Tang), CA70958 (N. Ikegaki), and CA85519 (N. Ikegaki).

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We thank Drs. Antonio Iavarone and Anna Lasorella for providing us with pBABE vector and a pBABE/*MYCN-ER* construct; Drs. Paul Lee, Neil Sidell, and William G. Woods for their critical reading of the article; and The Pediatric Oncology Group and Children's Cancer Group tumor banks for providing portions of tumor specimens used in this study.

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Cancer Res 2006;66:2826-2833.